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(21) International Application Number: PCT/US91/07158 (22) International Filing Date: 4 October 1991 (04.10.91) (30) Priority data: 592,801 4 October 1990 (04.10.90) US (60) Parent Application or Grant (63) Related by Continuation US 592,801 (CIP) Filed on 4 October 1990 (04.10.90) (71) Applicant (for all designated States except US): UNIVERSITY OF VIRGINIA PATENTS FOUNDATION [US/US]; 1224 West Main Street, Charlottesville, VA 22903 (US). (72) Inventors; and (75) Inventors/Applicants (for US only) : TAYLOR, Ronald, P. [US/US]; 108 Kerry Lane, Charlottesville, VA 22901 (US). SUTHERLAND, William, M. [US/US]; 8 Chestnut Lane, Box 167, Earlysville, VA 22936 (US). REIST, Craig [US/US]; 600 Brandon Avenue, #38, Charlottesville, VA 22903 (US). WRIGHT, Eleanor, L. [US/US]; Route 2, Box 79A, Earlysville, VA 22936 (US). WEBB, Donna [US/US]; 270 Colonnade Drive, University Heights, Apartment 18, Charlottesville, VA 22901 (US). LABUGUEN, Ronald [US/US]; 112B Lewis, Station #1, Charlottesville, VA 22904 (US).		(74) Agents: KELBER, Steven, B. et al.; Oblon, Spivak, McClelland, Maier & Neustadt, Fourth Floor, 1755 South Jefferson Davis Highway, Arlington, VA 22202 (US). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU*, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i>
(54) Title: PRIMATE ERYTHROCYTE BOUND MONOCLONAL ANTIBODY HETEROPOLYMERS (57) Abstract <p>The application relates to the use of antibodies in therapy. Monoclonal antibody heteropolymers are described which comprise a monoclonal antibody specific for the CR1 receptor on primate red blood cells covalently linked to a second monoclonal antibody specific for an antigen to be cleared from the circulatory system. The monoclonal antibody heteropolymers may be injected directly into a patient's circulatory system. Alternatively, red blood cells removed from a patient or a suitable donor may be contacted with the monoclonal antibody heteropolymers and then administered to the patient.</p>		

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Description

Primate Erythrocyte Bound Monoclonal Antibody Heteropolymers

Technical Field

5 This invention is directed to mammalian primate erythrocytes to which have been bound cross-linked monoclonal antibodies (heteropolymers) specific for both the erythrocyte complement receptor protein (CR1), and (a
10 2nd antibody bound thereto specific for) a circulating antigen. Methods of using these "franked" erythrocytes in diagnostic or assay methodology and therapeutic applications are also addressed.

Background Art

15 Mammalian primate erythrocytes (RBC's) have been identified as essential to the body's ability to clear antibody/antigen immune complexes from the blood. Specifically, the RBC receptor (CR1), known to be specific for certain activated complement proteins (C3b, C3bi and C4b), has been implicated as playing an
20 important role in the primate's defense against microorganism infection by facilitating the neutralization and clearance of certain pathogenic substances. Other evidence shows that the binding of these immune complexes to RBC's at the CR1 site provides
25 a vehicle for rapid clearance of potentially pathogenic immune complexes from circulation. Enhancement of phagocytosis and circulatory transport of immune complexes have both been advanced as mechanisms by which the RBC's function in this immune response have been
30 described. See, e.g., Nelson, Science 118, 733-737 (1953) and Hebert et al, Kidney Int., 31, 877-885 (1987). In any event, defects in aspects of this RBC clearance

method have been demonstrated to be at least statistically related to a number of diseases and are believed to progress various disease activities.

Notwithstanding the importance of this function of the RBC and the immune system, it is apparent that the RBC binding and clearance capacity therefore is confined to immune complexes recognized by the CR1 receptor, that is the immune complexes must contain large amounts of at least one of the activated Complement proteins C3b, C3bi, or C4b. Thus, the mammalian primate or human body has no normal capacity to take advantage of the clearance system provided by the RBC binding ability to remove antigens not complexed with the identified activated complement proteins. It remains an object of those of skill in the art to augment the natural capacity of the mammalian circulatory system to clear antigens through RBC binding ability to include the ability to bind immune complexes (antigen/antibody complexes) via CR1 to RBC's in the absence of activated complement proteins. These augmented RBC's would be useful both in a therapeutic sense, as well as in an assay mode to identify the presence or absence of specific antigens.

Disclosure of the Invention

Specific monoclonal antibody heteropolymers are prepared from Mabs specific to the CR1 RBC receptor and Mabs to at least one other antigen and are then heteropolymerized using established techniques, as reported in U.S. Patent Application Serial No. 07/592,801 filed October 4, 1990. This heteropolymer binds readily to RBC's in whole blood, in numbers in good agreement with the number of CR1 sites available. The RBC's, if franked in vivo with the heteropolymer, will then bind

th antigen for which the remaining Mab is specific. These RBC's then can act therapeutically by facilitating the neutralization and clearance from the circulation of the bound antigen.

5 Alternatively, if introduced into a blood sample, franked RBC's bind quite rapidly to the antigen for which the second Mab is specific, and can also be used to assay for the presence of that particular antigen. If necessary, labeling of the heteropolymer and/or the
10 antigen with, e.g., radioactive iodine, can facilitate bound RBC counts, and both qualitative and quantitative assessment of the antigen presence. Specifically, the heteropolymer-franked RBC's can be used in clinical assays for antigens in the circulation as demonstrated by
15 the following example: Franked RBC's would be added to the plasma or anti-coagulated blood and allowed to bind the putative target antigen. After a wash the presence of the antigen bound to the RBC's would be revealed using appropriately labelled (e.g. either with ^{125}I , or enzyme-
20 linked) second antibodies to the target antigen. Such assays can be either qualitative or quantitative.

RBC's removed and isolated may also be used as therapeutic agents. Once franked with heteropolymer, these RBC's can be reintroduced into the patient, where,
25 in the bloodstream, free antigen will be bound and immobilized on the RBC, and cleared in accordance with the body's RBC clearing mechanism, which has been identified but is not completely understood. The franked RBC's can be specific for known antigens, such as HIV
30 (the AIDS virus), or for substances which, if present in large amounts, can induce or aggravate disease states, such as low-density lipoproteins, or cause adverse biological effects, such as elevated hormone levels.

Given the general ease with which Mabs can be prepared for any known antigen, the variety of franked RBC's imaginable is unlimited. Any antigen or immunogen found in the bloodstream can be addressed by this therapeutic method.

In an alternative embodiment, RBCs are franked with a "cocktail" of several heteropolymers which, in addition to binding the target antigen, also bind to several distinct and non-overlapping sites on CR1 of the RBC.

10 These points are identified, by e.g., Mabs 1B4, HB8592, and 57F. Experiments have demonstrated that by using two or more non-overlapping Mabs for binding to CR1 on the RBC, the number of Mab heteropolymers that can be bound to a single RBC is increased in numbers in good agreement

15 with the number of available binding sites. This augments the capability of a relatively small number of RBC's to bind to a relatively larger amount of antigen, and can further facilitate removal of the antigen through the normal immune clearance system.

20 Brief Description of the Drawings

Figure 1 illustrates heteropolymer mediated binding of Human IgG to erythrocytes as compared to controls (unfilled columns in Fig. 1b). Figure 2 reflects heteropolymer mediated saturation of binding of DNP₅BGG

25 to human erythrocytes.

Figures 3 A and B reflect in vivo clearance of injected antigen pursuant to the claimed invention.

Figures 4A and B gives similar data for another test individual.

Figure 5A gives similar clearance data for an independent test in another individual.

Figure 5B traces the degree of change in ^{51}Cr label throughout a clearance test.

- 5 Figures 6A, 6B and 6C reflect clearance of heteropolymers of the claimed invention from the circulation of a Rhesus monkey.

Best Mode for Carrying Out the Invention

As noted above, the flexibility of the franked RBC's of this invention in addressing a variety of disease states is limited only by the varieties of different antigens present in or accessible to the circulatory system and to which Mab can be prepared. A variety of Mab heteropolymers have been prepared. In order to attach to the RBC, the antigen-specific Mab is cross-linked with a Mab to the RBC complement receptor, CR1. Methods of cross-linking these antibodies are known to those of skill in the art. In the examples set forth below, some cross-linked heteropolymers were prepared using N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) according to established, published procedures. For details as to this procedure, see, e.g., Karpovsky et al, J. Exp. Med. 160, 1686-1701 (1984); Perez et al, Nature, 316, 354-356 (1985) or Titus et al, Journal of Immunology, 139, 3153-3158 (1987). In an alternative embodiment, heteropolymers are formed by biotinylating a Mab, incubating the biotin-bearing Mab with an avidin or strepavidin molecule, and then introducing a second Mab, also bearing a biotin linker arm. The thus-formed heteropolymer is a Mab-biotin-avidin/strepavidin-biotin-Mab sandwich. Other procedures are known to those of

rdinary skill in th art. A full listing of Mab heter polymers prepar d appears in Table 1, infra.. Prototype antigens select d for targeting through preparation of appropriat heteropolymers include
5 dinitrophenylated bovine gamma globulin (DNP₅₅BGG), and human IgG. Both antigens and heteropolymers were iodinated by the IODOGEN method (Fraker et al, Biochem. Biophys. Res. Commun. 80, 849-857 (1978). Iodination provides one protocol for assay utilization, but of
10 course need not be practiced for the therapeutic aspects of the claimed invention.

Assays for CR1 levels on isolated RBC's followed standard methods, revealing about 200-500 epitopes per RBC, as bound to by anti-CR1 Mabs 1B4, 3D9 and HB8592.

15 Details regarding RBC binding, binding kinetics and observed values follow below.

Examples:

1) Direct sensitization and binding isotherm analyses: Between 0.1 and 1.0 ml of a 10%-50% dispersion
20 of washed RBC's in bovine serum albumin/phosphate buffered saline (BSA-PBS) were reacted for 1 hr at room temperature, with shaking, with varying amounts (10-50 ul) of a dilution of one or more of the heteropolymers. The RBC's were then washed 3 times in BSA-PBS (to remove
25 excess unbound heteropolymer) and, after reconstitution in either BSA-PBS or normal human serum (for experiments with ¹²⁵I-human IgG and DNP₅₅BGG, respectively), mixed with a small volume of ¹²⁵I-probe. After a further incubation (usually 1 hr at room temperature, with
30 shaking), RBC-bound and free ¹²⁵I-antigens were separated by either of two procedures: RBC's were spun through oil

(typically 150 μ l of reaction mixture was layered on 200 μ l of a dibutyl-diethyl phthalate mixture), and simply processed by two cycles of centrifugation and washing in BSA-PBS. RBC-associated ^{125}I counts were quantitated in a Beckman 5500 gamma counter.

2) "Whole Blood" binding kinetics: a) In most procedures blood was drawn into Alsever's and centrifuged. A portion of the supernatant was removed, and after the blood cells were redispersed to a final hematocrit of 50%, a small amount of ^{125}I -DNP₅₅BGG antigen was added. Varying amounts of heteropolymer were added directly to aliquots of these "whole blood" dispersions containing ^{125}I -DNP₅₅BGG, and incubated with shaking at 37°C. RBC associated ^{125}I counts were determined at varying time points after centrifugation and washing steps. Selected aliquots of the reaction mixtures were also centrifuged through percoll to confirm that only RBC's (not white cells) bound the ^{125}I -antigen. In some of these "whole blood" experiments, instead of using Alsever's as an anti-coagulant, blood was drawn into EDTA or citrate and used at once in a similar manner. A few comparable "whole blood" experiments were also performed with ^{125}I -human IgG as the target antigen. In these experiments washed RBC's were dispersed in BSA-PBS, to avoid the potential confounding effect of endogenous serum-associated IgG. b) In other kinetic experiments one volume of RBC's was fracked with saturating amounts of the heteropolymer, and after three washes was added to 10 volumes of anti-coagulated blood containing ^{125}I -DNP₅₅BGG, and incubated at 37°C. Aliquots of the dispersions were processed periodically to determine RBC-associated ^{125}I counts.

Direct binding of ^{125}I -heteropolymers to a number of

matrices was determined in procedures analogous to those described above. For example, duplicate aliquots of 100 μ l of ^{125}I -heteropolymer #4 (see below) were incubated for one hour at room temperature, with shaking, with either
5 100 μ l of a 50% dispersion of human RBC's, or 100 μ l of a 33% dispersion of human IgG-Sepharose. Samples were then subjected to two cycles of centrifugation and washing and the levels of matrix-bound ^{125}I counts were determined. Direct binding to human RBC's of the ^{125}I -heteropolymers
10 was also determined as a function of time at 37°C.

Control experiments tested for the specificity of antigen binding by heteropolymer treated RBC's and verified the requirement for CR1. These experiments included the use of heteropolymer-treated sheep RBC's
15 (which lack CR1), naive (untreated) human RBC's, and excess homologous monomeric Mabs (in ascites fluid) which blocked the action of the heteropolymers.

Results

Preparation and Initial Characterizations of

20 Heteropolymers. We prepared a number of heteropolymers by SPDP cross-linking, and examined the ability of these heteropolymers to react with human RBC's and facilitate binding of specific antigens. Preliminary data (Table 1), using mixtures of saturating amounts of unfractionated
25 material (containing heteropolymers and non-cross-linked monomers), demonstrated specific RBC-associated binding of the ^{125}I -antigens. An excess of ^{125}I -antigen was used in order to determine the maximum number of ligands bound per RBC. For each heteropolymer mixture the results
30 (Table 1) are in good agreement with the typical number of CR1 epitopes (200-500) recognized by the anti-CR1 Mabs.

Heteropolymer mixture #1 can facilitate binding via two noncompeting Mabs to CR1, 1B4 and HB8592. This mixture can, therefore, place approximately twice as many anti-IgG heteropolymers on the RBC's as a heteropolymer containing only one anti-CR1 Mab. The maximum ^{125}I -human IgG bound to such "doubly-franked" RBC's is nearly equal to the sum of the ^{125}I -IgG bound to RBC's franked with two individual components of the mixture (Table 1); this illustrates the principle of additivity. Dose-response experiments with heteropolymer #1 and other heteropolymers (Table 1, and see below) confirm that RBC binding of both heteropolymer and ^{125}I -antigen is saturable. "Background" binding of antigen to naive RBC's is low, and use of heteropolymers with "irrelevant" specificities for the target ligands (e.g. 8E11 (anti-C3b) X HB8592) gave no binding (Table 1).

Binding Isotherms with Isolated Heteropolymers.

Heteropolymer mixtures were further purified by gel permeation chromatography, and the highest molecular weight subfractions (ca. corresponding to trimers and larger species) were used to quantitate binding (Figures 1 and 2). In these experiments binding of ^{125}I -antigens to franked RBC's was determined, after two cycles of centrifugation and washing with BSA-PBS, by direct counting of the RBC pellets.

At saturating input of heteropolymer, the maximum number of antigen molecules bound per RBC is in good quantitative agreement with our initial survey results using unfractionated heteropolymer mixtures and centrifugation through oil to separate bound from free ^{125}I -antigen. These experiments confirm that binding is saturable, since use of excess quantities of a single heteropolymer or ^{125}I -antigen does not increase binding

beyond the saturation level (typically 200-1000 antigens per RBC, Figures 1 and 2). Analysis of results with blood from two donors (Figure 1a and 1b) demonstrates that maximum binding reflects the number of CR1 epitopes per RBC characteristic of the individual donor. The principle of additivity is also illustrated in experiments in which RBC's were franked with a combined mixture of two heteropolymers (Figure 1a). The combined action of the two heteropolymers in facilitating binding of the ^{125}I -antigen is close to the sum of the action of each species individually.

Bi-specificity of the heteropolymers was demonstrated in inhibition experiments using an excess of homologous monomeric Mab. Our goal was either to block binding of heteropolymer to RBC's (using an appropriate anti-CR1 Mab to thus preclude binding of ^{125}I -DNP₅₅BGG), or to inhibit directly binding of ^{125}I -DNP₅₅BGG to franked RBC's (using the appropriate monomeric anti-DNP Mab). In all cases more than 90% of specific binding was reduced by these procedures (Figure 1b). Sheep RBC's lack CR1, and as anticipated, heteropolymers directed against CR1 do not facilitate binding of the ^{125}I -antigen to sheep RBC's (Figure 1b). Finally, the dual specificities of two of the heteropolymer mixtures was confirmed by labelling them with ^{125}I and examining their binding to human RBC's and to a Sepharose 4B matrix containing their respective target antigens (Table 2). The results demonstrate that the isolated polymers bind to both of their respective matrices, and also confirm that their direct binding to human RBC's is rapid at 37°C.

TABLE 1

**Survey of Cross-Linked Mab Heteropolymer Mixtures in
facilitating**

Antigen Binding to RBC's

A. Binding of ^{125}I -Human IgG

Hetero Polymer #	Mab #1 (Specificity) X Mab #2 (Specificity) *	Molecul s** IGG Bound/ RBC
1	1B4(anti-CR1) X HB43 (anti-IgG) and HB8592 (anti-CR1)	1150
2	HB43(anti-IgG) X HB8592 (anti-CR1)	601
3	HB43(anti-IgG) X 1B4(anti-CR1)	492
4	HB43(anti-IgG) X HB8592(anti-CR1)	357
5	HB43(anti-IgG) X 1B4(anti-CR1)	479
6	HB43(anti-IgG) X 3D9(anti-CR1)	355
7	HB43(anti-IgG) X 57F(anti-CR1)	387
Control	8E11(anti-C3b) X HB8592(anti-CR1)	-2

B. Binding of ^{125}I -(DNP)₅₅BGG

<u>DNP₅₅BGG</u>			Molecules Bound/ RBC
Heteropolymer	Mab #1 (Specificity) X Mab #2 (Specificity) *		
8	3D9(anti-CR1) X 2A1(anti-DNP)		191
9	3D9(anti-CR1) X 23D(anti-CR1)		243
10	23D1(anti-DNP) X HB8592(anti-CR1)		129
11	23D1(anti-DNP) X 1B4(anti-CR1)		255
12	23D1(anti-DNP) X 3D9(anti-CR1)		196
13	HB8592(anti-CR) X 2A1(anti-DNP)		95
14	HB8592(anti-CR1) X 23D1(anti-DNP)		133
15	1B4(anti-CR1) X 23D1(anti-DNP)		279
16	1B4(anti-CR1) X 2A1(anti-DNP)		236
Control	8E11(anti-C3b) x HB8592(anti-CR1)		-11

C. Demonstration of Saturation of Binding with Heteropolymer #1 and ^{125}I -IgG

Relative Heteropolymer Concentration	Relative ^{125}I - Human IgG Concentration	Molecules** IgG Bound RBC
5	5	994
5	1	868
5	0.2	343
1 ***	1 ****	777
0.2	1	205

Table 1 of notes

For each heteropolymer listed, the first Mab was reduced with dithiothreitol (after reacting with SPDP) and then coupled to the second SPDP-reacted Mab. Heteropolymers #2 and #4 represent preparations with HB8592 purified via protein G and octanoic acid-50% saturated ammonium sulfate, respectively. Heteropolymer #1 was prepared by simultaneously reacting a cocktail of SPDP-coupled and reduced 1B4 and HB8592 with SPDP-coupled HB43.

** Binding was determined by centrifuging RBC's through oil. Background binding to naive human RBC's was 40 human IgG and 60 DNP₅₅BGG per RBC respectively, and was subtracted to give the net specific binding reported. In parts A and B predetermined saturating inputs of both heteropolymer and ¹²⁵I-antigen were used.

*** "1" corresponds to 3.0 ug/ml of heteropolymer in a 12.5% hematocrit.

****"1" corresponds to 0.92 ug/ml ¹²⁵I-human IgG in a 12.5% hematocrit.

TABLE 2

**Binding of ^{125}I -Lab 11 d Heteropolymers
to Human RBC's or to Sepharose Coupled Ligands**

	<u>% Bound *</u>	
	<u>Human</u> <u>RBC's</u>	<u>IgG (or DNP)-</u> <u>Sepharose</u>
#4, unfractionated mixture (HB43(anti-IgG) X HB8592(anti-CR1))	45 \pm 5	55 \pm 5
#4, isolated polymer fraction	65 \pm 3	86 \pm 5
#11, unfractionated mixture (23D1(anti- DNP) X 1B4 (anti- CR1))	34 \pm 6 **	86 \pm 5
#11, isolated polymer fraction	72 \pm 2 ***	85 \pm 5

Table 2 footnotes

- * Bound after incubation (with an excess of binding matrix) for one hour at either room temperature (Sepharose samples) and/or 37 °C (RBC's were examined at both temperatures). IgG Sepharose was used as the binding matrix for heteropolymer #4, and DNP-Sepharose (containing a dinitrophenylated Mab to IgM) was used for #11. All samples were corrected for background binding (5% or less) to sheep RBC's or naive (unreacted) Sepharose.
- ** Binding was 30% and 32% respectively after incubation for either two or five minutes at 37°C.
- *** Binding was 57% and 69%, respectively, after incubation for either two minutes or five minutes at 37°C.

PRIMATE STUDIES:**Materials and Methods**

Mab and Heteropolymer Preparation. Purified Mab to CR1, the DNP group, and human IgG were biotinylated following published procedures Wilchek et al, Meth. Enzym., 184, Avidin-Biotin Technology (1990) using the "long-arm" biotinylating agent biotinyl-N-hydroxy succinimide (Vector Laboratories, Burlingame, CA) at molar inputs of biotin to Mab between 5 to 1 and 20 to 1. The Iodogen method Fraker et al, Bioc & Biop. Review, 80:849 (1978) was used to label both naive and biotinylated Mab with ^{125}I . DNP₅BGG Little et al, Neth. Immun. Immunochem, 1:128 (1967), was similarly labelled with either ^{125}I or ^{131}I . Preparation of soluble cross-linked heteropolymer (HP) which specifically bound to primate E was accomplished by first incubating (at 37°C for 30 min) a biotinylated Mab to CR1 (either 1B4₁₀ or E11₁₀) with excess strepavidin (SA) (Gibco BRI, Gaithersburg, MD). Subsequently a biotinylated Mab to the DNP group (23D1₅) was incubated with this complex, and the resultant HP was then used without further purification. Detailed dose response tests were performed to determine the optimum inputs of biotinylated first Mab (anti-CR1), SA, and biotinylated second Mab (antiDNP) in the generation of soluble HP which bound to human and other primate erythrocyte (E) and facilitated antigen (Ag) binding. In all cases we verified that the HP used in these studies neither directly bound to, nor facilitated Ag binding to sheep E, which lack CR1 (22).

RIA. Varying inputs of radiolabelled HP (either the anti-CR1 Mab or anti-DNP Mab portion of the HP was radiolabelled) were incubated at 37°C for 5-15 min with a

50% dispersion (in homologous serum) of human or primate E [a "whole blood" simulation Ross et al, J. Immunol., 135:2005 (1985)]. Binding was determined by counting the E pellets after centrifugation and washing. Then quantitate HP-mediated binding of Ag (DNP₅BGG) to E, the radiolabelled Ag was first added to the "whole blood" dispersion, and after varying amounts of HP were added, the samples were processed similarly. In some in vitro Ag-binding experiments freshly drawn anti-coagulated blood was used, and Ag binding via the HP was also demonstrable.

The number of CR1 epitopes per E was determined by incubating an excess of ¹²⁵I-labelled Mab to CR1 with isolated and washed E followed by one of two procedures for separation of E-"bound" from "free" Mab: 1) the incubation mixtures were layered on dibutyltinonyl phthalate mixtures, and after a centrifugation step the samples were frozen and the E pellets were cut off or counted; 2) alternatively, the E dispersions were simply pelleted, and after washing, the E pellets were counted. In all RIA, sheep E controls were included to provide a background correction, which was less than 10% of specific binding to human E.

In Vivo Experiments. We followed protocols similar to those we previously reported for studying clearance of IC from the circulation of both non-primates and primates. See, e.g., Taylor et al, J. Immunol. 139:1240 (1987). The doses of Ag and HP chosen in these experiments were optimized based on titration experiments performed in anti-coagulated whole blood samples from both humans and monkeys: 1 ml of radiolabelled DNP₅BGG (3-25 µg) in PBS was infused within 30 seconds into the catheterized saphenous vein of a 1 kg squirrel monkey

sedated with ketamin and maintained under anesthesia with halthane. After 20-35 min, 1 ml of HP (0.25-1.0 mg, either radiolabelled or unlabelled) was injected. Multiple blood aliquots (anti-coagulated in citrate) were collected over the entire course of the experiment to determine both the "endogenous" clearance rate of the Ag, and the effect of the HP on its clearance and E binding. Within 10 min of collection each blood sample was centrifuged and the cell pellet washed and counted to determine binding. The supernatants were combined and counted, and aliquots were examined to determine net 5% TCA-precipitable counts. Initially after injection, ^{125}I and ^{131}I counts recovered in the plasma supernatants were > 95% and 85% TCA-precipitable, respectively. Finally, selected blood samples were centrifuged through percoll gradients (which only allowed pelleting of the E). This procedure verified that cell-associated counts were only due to E binding. The same basic methodology was followed in our experiments with 10 kg rhesus monkeys, except 0.25 mg of HP was injected.

Quantitative Analyses. The specific activities of all proteins were determined and used to calculate the number of molecules bound per E for both in vivo and in vitro experiments. In the case of double or triple label experiments (^{125}I and ^{131}I , or both iodine labels and ^{51}Cr) the overlap of different labels was determined in calibration experiments and corrected counts were calculated for each isotope.

Assays for an Immune Response. We used a solid phase "Ag capture" assay, Khazaeli et al, J. Biol. Resp. Med., 91:178 (1990) to determine if the animals developed an immune response to the injected agents (biotinylated mouse IgG, SA, and DNP₅BGG). This assay protocol

involves adhering the Ag to a solid matrix, adding a 10-20 fold dilution of the serum to be tested, and then adding radiolabelled Ag as a probe. Specific binding of antibody to solid phase Ag then allows capture of solution phase radiolabelled Ag. Positive controls in this assay included goat anti-mouse IgG, biotinylated human IgG, and mouse anti-DNP Mab (23D1), respectively. Alternatively, in order to increase the sensitivity of the assay, we "developed" with ^{125}I -Mab HB43, an anti-human IgG Mab which cross reacts with monkey IgG. This "direct" solid phase RIA, is considerably more sensitive for detecting a weak immune response (see below).

Results

In vitro preliminary studies in squirrel monkeys. We focused on the use of anti-CR1 Mab 1B4 in these clearance studies because it is known to block the "active site" (the C3b-binding site) of human CR1 (30) and therefore should bind close to the most "biologically relevant" site in terms of in vivo recognition by fixed receptors on liver or spleen cells. Also, large amounts of this Mab can be prepared from the hybridoma cell line. However, even though there are a moderate number of CR1 epitopes on the squirrel monkeys' E, defined by Mab E11, there are far fewer binding sites for Mab 1B4 on the monkey E compared to the number found on human E (Table 3A). Binding of Mab 1B4 to squirrel monkey E also appears to be of lower avidity than binding to human E, as evidenced by the relatively large reduction in net binding to the monkey E when the E are processed through a wash step, rather than simply by centrifugation through oil (Table 3A).

Construction of soluble, cross-linked multivalent A/B HP with biotinylated Mab 1B4₁₀ considerably enhanced binding of this Mab to squirrel monkey E and caused an increase in binding to human E as well (Table 3B). Use of a radiolabel on the "second" (anti-target Ag) biotinylated Mab 23D1₅ of the HP confirms that the A/B system does fix both biotinylated Mab to the primate E (Table 3B). The specificity of binding is confirmed by several experiments: "Background" binding of the HP to sheep E (lacking CR1) is much lower; and, in the presence of excess monomeric Mab 1B4 (in ascites), HP-mediated binding to human and squirrel monkey E is almost completely eliminated (Table 3C). The results also indicate that more HP-associated, biotinylated 23D1₅ Mab is bound to the E than the biotinylated 1B4₁₀ Mab, which is probably a consequence of amplification due to the multivalent nature of the A/B system. Finally, binding of the biotinylated 23D1₅ Mab to primate E is reduced to background levels (the level seen for sheep E) unless both biotinylated 1B4₁₀ and SA are used for construction of the HP (data not shown).

We used A/B HP prepared with biotinylated preparations of Mab 23D1 and either Mab 1B4 or Mab E11, to facilitate binding of the target Ag DNP₅BGG to primate E (Table 4) in a "whole blood" experiment analogous to those we have reported using SPDP-HP. It can be seen that comparable binding is obtained for HP constructed with either Mab E11₁₀ or 1B4₁₀. We chose DNP₅BGG (a protein with a low degree of dinitrophenylation) as binding substrate for the *in vivo* experiments because it has been reported that a high degree of protein dinitrophenylation leads to rapid clearance from the circulation, even in the absence of specific antibody. The affinity of Mab 23D1 for the DNP group is only ca.

5×10^6 L/M, which is consistent with the moderate degree of binding (20-40%) of the DNP₅BGG by E opsonized with a HP containing 23D1₅. A similar level of target Ag binding is demonstrable in vivo (see below).

- 5 In vitro preliminary studies in cynomolgus and rhesus monkeys. The level of detectable E11 and 1B4 epitopes on the E of these primates was higher than that detected for the squirrel monkeys (Table 5). In these experiments we prepared A/B HP using a "second" Mab, HB43, specific for
- 10 human IgG. In vitro binding of these HP to monkey E is quantitated in Table 5.

In vivo clearance kinetics in squirrel monkeys.

- Injection of ¹²⁵I-DNP₅BGG into the squirrel monkey leads to rapid clearance of a fraction of the injected Ag
- 15 counts within the first 15 min, followed by a slower phase of clearance (Figure 3). After 20 min we injected a 1B4₁₀/SA/23D1₅ HP specific for both monkey CR1 and the DNP group. Within 10 min ca. 20% of the circulating Ag counts became E bound. Consistent with the uptake of
- 20 counts by the E is a comparable loss of counts from the plasma. It is also evident that the majority of E-associated counts are then cleared rapidly from the circulation because a significant number of these counts do not return to the plasma phase. Finally, those plasma
- 25 counts that did not become E-bound continued to be cleared at a relatively slow rate.

- Clearance of both target Ag and HP were then followed through a double label experiment. The data in Figure 4A again demonstrates a rapid initial phase of Ag
- 30 clearance followed by an approximate plateau and subsequent slow removal of plasma counts from the circulation. It should also be noted that less than 3%

of the counts are E-bound before the HP is injected (the first 25 min of the experiment). Upon injection of HP there is a drop in Ag-associated plasma counts which is ca. equal to the generation of E-associated counts (a maximum of 31% of the Ag was bound to the E). It is also evident that the majority of injected HP binds rapidly to the E (a maximum of 60%), and that subsequently both E-bound HP and Ag are cleared at approximately the same rate. The co-clearance of E-bound HP and Ag is also illustrated in a comparable experiment performed on a different squirrel monkey (Figure 4B). We also conducted an independent double label experiment similar to that depicted in Figure 4A, except the ^{125}I label was on the 23D1₅ Mab (Figure 5A). The rate of clearance of E-bound HP and Ag was again approximately the same, and although maximum binding of Ag was 35% (comparable to earlier experiments), the maximum level of HP binding (defined by the ^{125}I label on the anti-DNP 23D1₅ Mab) was reduced to 40%.

In order to address the potential problem associated with HP-mediated lysis of the E we co-injected ^{51}Cr labelled E along with ^{131}I -labelled DNP₅BGG, and followed both labels. After 35 min ^{125}I -labelled HP was injected and the clearance of all 3 radiolabels was monitored. Clearance of the ^{131}I -labelled DNP₅BGG (ca. 25% maximum E binding) followed the same trends seen in previous experiments (data not shown). Throughout the course of the experiment the ^{51}Cr label remained associated with the E pellet, and showed no significant change before or after injection of the HP (Figure 5B). In this experiment, 50% of the ^{125}I -labelled HP bound to the E, and the E-associated counts were cleared rapidly. Also, in agreement with the results shown in Figure 2, the fraction of HP that remained in the plasma was cleared at

a much slower rate.

In vivo clearance kinetics in rhesus monkeys. The level of HP binding (per E) to the squirrel monkey E was much higher than the level of Ag binding, and it is unlikely the Ag itself facilitated clearance of the majority of E-bound HP. However, it is important to demonstrate that the present results can be generalized to other primates and other Ag systems. For these reasons we used the A/B system to prepare HP with biotinylated Mab 1B4₁₀ and human IgG₅, or with biotinylated Mab E11₁₀ and HB43₅ (an anti-human IgG Mab), and injected these HP into rhesus monkeys (Figures 6A and 6B). In these experiments we followed the HP with a radiolabel on the "second" antibody, rather than on the anti-CR1 Mab. The results clearly indicate that both these HP bind to the primate E and are then cleared rapidly. Once again the fraction of HP that did not bind to the E and remained in the plasma was cleared at a much slower rate. Consecutive experiments were performed on the same animals by injecting a second HP one hr after the first HP (Figure 6C). The overall clearance of the second HP from the animal's circulation was similar to that seen for the first injected HP.

We measured CR1 on E of the rhesus monkeys from blood aliquots obtained before and during these experiments, and the level remained constant (approximately equal to the levels reported in Table 5) during the course of the procedure. Finally, we tested these HP for complement activation and C3b capture after binding to human E in homologous serum. In some instances there was a low level of C3b binding to the E, but in other cases (e.g., the 1B4/human IgG HP) we could not detect any C3b bound to the E above background

1 v ls.

In preliminary experiments (performed 3 weeks prior to those discussed above) we injected rhesus monkeys with a "partial HP" containing radiolabelled-biotinylated anti-CR1 Mab and SA only (lacking the "second" biotinylated antibody) in order to determine if this "partial" HP could bind E and be cleared from circulation. Although binding of this complex to E was approximately the same as that of a complete HP (1B4₁₀/SA/23D1₅), no significant short term clearance was observed, but after 3 weeks all counts had been cleared from circulation.

Development of an immune response. We conducted a total of 3 similar experiments on each of 2 squirrel monkeys over a period of 6 weeks. In order to determine if the squirrel monkeys developed an immune response to any of the injected materials (biotinylated mouse Mab, SA, or DNP₅BGG) we performed solid phase "Ag capture" assays on serum samples taken from the animals before, during, and after the experiments. The results of this assay indicate that there was no demonstrable immune response in any of the squirrel monkeys over a period of 10 weeks. However, by assaying with the more sensitive "direct" solid phase RIA (see Materials and Methods), we found that squirrel monkey 417 (but not 899) exhibited a rather weak, but demonstrable immune response to mouse IgG (but not SA or DNP₅BGG). The immune response was detected after the first experiment and increased slightly with time. Dose response experiments using the "direct" RIA indicate the binding capacity of a 5-fold dilution of serum from squirrel monkey 417 was comparable to that of a 10,000 fold dilution of goat antimouse IgG.

Two clearance experiments were performed on the rhesus monkeys, with a 3 week interval between experiments (see above). The "Ag capture" assay revealed no immune response to biotinylated mouse IgG during this 5 time period. However, there was weak but measurable binding of mouse IgG by sera from both rhesus monkeys 2 weeks after the second experiment (data not shown). Using the more sensitive "direct" solid phase RIA (probing with ^{125}I -HB43) indicated the rhesus monkeys had 10 developed a weak but demonstrable immune response to biotinylated mouse IgG by the start of the second clearance experiment. The titer of anti-mouse antibodies peaked 2 weeks after the second experiment and persisted in the circulation for more than 2 months. At the peak 15 level (day 36), dose response experiments indicated that binding of the ^{125}I -HB43 probe corresponded to a titer of approximately 0.5% of that of a positive control, goat anti-mouse IgG (Table 6).

Table 3. Binding of anti-CR1 Mab and HP to Squirrel Monkey and Human E.

A. Direct CR1 Measurements ^a						
Molecules Bound per E ^b						
mAb	Binding Assay	S. Monkey 899	S. Monkey 417	S. Monkey 413	Human 1	Human 2
E11	Oil	310	280	290	578	510
E11	Washed Pellet	210	210	230	480	410
1B4	Oil	50	30	50	340	330
1B4	Washed Pellet	10	5	20	280	260
1B4 ₁₀ ^c	Oil	55	30	60	310	310
1B4 ₁₀	Washed Pellet	10	4	20	240	230

B. "Whole Blood" Solution Phase Binding of HP ^{a,d}					
Molecules Bound per E ^b					
HP	S. Monkey 899	S. Monkey 417	S. Monkey 413	Human 1	Human 2
¹²⁵ I-1B4 ₁₀ /SA/23D1 ₅	640	470	600	1090	780
1B4 ₁₀ /SA/ ¹²⁵ I-23D1 ₅	4700	2330	5220	7460	4660

C. Inhibition of "Whole Blood" Solution Phase Binding by 1B4 Ascites					
Molecules Bound per E ^{a,e}					
HP	Inhibitor	S. Monkey 417	S. Monkey 413	Human 1	Human
¹²⁵ I-1B4 ₁₀ /SA/23D1 ₅	----	430	350	510	1000
¹²⁵ I-1B4 ₁₀ /SA23D1 ₅	1B4 Ascites ^f	54	43	60	-5

Footnot s f r Tabl 3

- a) Th uncertainti s in the reported valu s average $\pm 5\%$. In all cases contr ls with sheet E (which lack CR1) wer us d to subtract the background baseline level due to nonspecific binding.
- b) E were separated from excess iodinated Mab by either spinning through an oil cushion or by pelleting and washing the E.
- c) The subscripts refer to the molar input of biotin to Mab in the biotinylation reaction.
- d) HP were assembled in solution and added to a dispersion of E in serum containing 0.01M EDTA, and bound molecules (Mab) were determined by counting the washed E pellets.
- e) Half as much HP was incubated per E in part C compared to part B.
- f) 1B4 ascites fluid was pre-incubated with the E in part C. Irrelevant ascites from an anti-C3b Mab gave the same results as the sample lacking inhibitor.

Table 4. HP-mediated Binding ("Whole Blood" Solution Phase Assay) of ^{125}I -DNP₅BGG to Squirrel Monkey and Human E.*

HP	% Bound				
	S. Monkey 899	S. Monkey 417	S. Monkey 413	Human	Sheep
E11 ₁₀ /SA/23D1 ₅	28	38	20	34	1
1B4 ₁₀ /SA/23D1 ₅	42	44	14	38	1
(Naive E)	2	2	2	1	1

Molar impact of ^{125}I -DNP₅BGG corresponded to 37 molecules per E.

Table 5. Binding of Anti-CR1 Mab and HP to Rhesus Monkey E and Cynomolgus Monkey E.

<u>Mab^b</u>	<u>Molecules Bound Per E^a</u>		
	<u>Rh Monkey 4F</u>	<u>Rh. Monkey 941</u>	<u>Cy Monkey 69</u>
E11	1760	1580	1480
1B4	230	140	110
<u>HP^c</u>			
1B4 ₁₀ /SA/ ¹²⁵ I-HB43 ₅	710	700	480
E11 ₁₀ /SA/ ¹²⁵ I-HB43 ₅	620	610	460

- a) See footnote a, Table 3.
- b) Binding was determined by centrifugation through oil.
- c) A relatively low input of HP per E was used. Binding values in a comparable experiment for an E Sample from a human were 1330 and 810 molecules per E for the HP containing 1B4₁₀ and E11₁₀, respectively.

Tabl 6. Representative "Direct" Solid Phase RIA for
Detecting an Immune Response to Biotinylated
Mouse IgG in Rhinus Monkey 941.

A. Binding as a Function of Time^a

<u>Serum Source^b</u>	<u>\pm ¹²⁵I-HB43 Bound to Solid Phase Matrix</u>	
	<u>1B4₂₀^c</u>	<u>Mouse IgG^c</u>
pre-immune serum	0.8	0.8
Day 21	6.9	6.1
Day 36	9.5	15.3
Day 49	4.7	8.0
Day 80	1.9	2.9
goat anti-mouse IgG (Control)	23.2	22.2

B. Relative Titer (Day 36)

<u>Serum and Dilution</u>	<u>\pm ¹²⁵I-HB43 Bound to Solid Phase Matrix^d</u>	
	<u>1B4₂₀^c</u>	<u>Mouse IgG^c</u>
Serum/20	12.2	15.6
Serum/80	4.0	4.2
goat anti-mouse IgG/200	36.5	37.0
goat anti-mouse IgG/1000	21.5	30.0
goat anti-mouse IgG/5000	4.6	9.4

- a) The first injection (of the "partial" HP) was on day 1, and on day 21 the "complete" HP was injected.
- b) All sera were examined at a 10 fold dilution.
- c) Either 1B4₂₀ or mouse IgG was used to first coat the plates (see Materials and Methods).
- d) A different input of ¹²⁵I-HB43 was used in Part B compared to that in Part A.

METHODS OF USE

Th frank d RBC's d scribed above have immediat application in a variety f r s arch, clinical diagnostic, or therapeutic uses. The most important are

5 therapeutic uses, which can include (1) using a franked RBC of the invention with specificity to an antigen such as HIV to clear free antigen from the blood of a human or primate patient, (2) using a franked erythrocyte with a specific Mab to clearfor a non-immunogenic but

10 potentially "pathogenic" target such as LDL which has been linked to atherosclerosis and (3) using a franked erythrocyte with Mab specificity for the natural ligand of CR1 (such as C3b) where the number of naturally occurring receptors in an individual patient has

15 decreased, such as in systemic lupus erythematosus. Of course, the specific antigens or antibody targets identified above are exemplary only, and virtually any circulating microorganism, virus, compound and the like to which a Mab can be prepared can be subject to

20 therapeutic treatment through the invention.

The franked erythrocytes may be prepared and introduced for therapeutic use in either of three methods. First, the bi-specific heteropolymer comprised of at least two cross-linked Mab, one specific for the

25 RBC and the other for the antigen, may be introduced directly to the bloodstream through inoculation. Alternatively, a small amount of RBC's can be extracted from the patient, and bound to the heteropolymer in sterile in vitro conditions and then reintroduced into

30 the patient. Finally, in cases of low CR1 or low RBC disease states, franked erythrocytes from a compatable heterologous matched blood donor can be used. In any of the above examples, a "cocktail" of several

heteropolymers (see results section) can be used. Given
th high binding capacity f th het ropolymer t the
RBC, direct injection of the heter p lymer can be as
ffective as in vitro preparation of the franked
5 erythrocyte, followed by inoculation.

In addition to taking advantage of the body's
natural defenses by augmenting the natural immune defense
system, binding the heteropolymer to the RBC's (either in
vitro or in vivo) may reduce or remove any
10 "immunogenicity" that would be characteristic of Mabs
prepared from mouse hosts and the like. In fact,
currently, the vast majority of monoclonal antibodies are
produced in mice. Mab treatment thus suffers, at least
to some degree, from the body's natural immune response
15 against the mouse Mab which would thus prevent the Mab
from binding to its target antigen. As the number of
heteropolymers bound per RBC is relatively small (below
about 500) the Mab itself may not be recognized as
foreign, and the host immune response may not be
20 triggered, or at least, will be significantly reduced.
This appears to be born out by the results reported. Use
of available human Mabs to prepare heteropolymers should
also eliminate any host immune response.

The dosage and treatment regimen will vary from
25 antigen to antigen, individual to individual, and disease
state. In general, these can be determined on an
empirical basis. An extreme minority of available RBC's
may be used effectively in conferring therapeutic
treatment. This is due in part to the vast numbers of
30 RBC's present in the blood, in contrast to most antigens.
As an example, the level of HIV which circulates free in
the blood has been suggested as the most cytopathic form
of HIV. High levels of HIV in the circulation appear to

correlate with disease activity. Yet, this level ranges betw en 1,000 and 50,000 virus particles per ml. Ho et al, New England Journal of Medicine, 321, 1621-1625 (1989), Coombs et al., ibid, 1626-1631. In contrast, th

5 number of RBC per ml in circulation is many orders of magnitude greater, and accordingly, even a small minority of available RBC's treated according to the claimed invention should be sufficient to confer therapeutic treatment, given the appropriate anti-CR1/anti-HIV

10 franked RBC. As exemplary levels only, in the treatment of AIDS, an intravenous administration of no more than 1-4 mg of appropriate heteropolymer should be sufficient to frank the patients' RBC's for quantitative binding of circulating HIV. With sufficiently high avidity anti-HIV

15 antibodies in the heteropolymer (easily achieved by standard methods) it should be possible to use considerably less heteropolymer (μ g amounts). See the "Detailed Calculations Section" for a more complete analysis of this problem. Alternatively, if the RBC's of

20 the patient are first removed and franked with heteropolymer and then re-injected, the dose administration of franked erythrocytes would be considerably less than 1 "unit" (1 pint) of blood. Use of ca. 50-100 ml of franked RBC's (a few % of total

25 circulating RBC's) should be adequate. The low levels needed are a consequence of the fact that even under conditions of high disease activity the concentration of infectious agent in the blood (e.g. HIV) is many orders of magnitude lower than the concentration of RBC's.

30 This invention has been described by reference both to generic description and specific embodiment. Examples provided are not intended to be limiting unless so specified, and variations will occur to those of ordinary skill in the art without the exercise of inventive

faculty. Th invention embraces these alternatives, save for the limitations imposed by th claims s t f rth below.

Claims

1 1. A mammalian erythrocyte bound to a first
2 monoclonal antibody at a receptor site for which said
3 first monoclonal antibody is specific, said first
4 monoclonal antibody being cross-linked to a second
5 monoclonal antibody specific for an antigen present in
6 the mammalian primate circulatory system.

1 2. The erythrocyte of Claim 1, wherein said
2 erythrocyte bears, on its surface, up to 1000 of said
3 first monoclonal antibodies bound to said second
4 monoclonal antibody.

1 3. The erythrocyte of Claim 1, wherein said
2 erythrocyte receptor site is the CR1 protein.

1 4. The erythrocyte of Claim 1, wherein said antigen
2 is selected from the group consisting of a virus,
3 microorganism or toxic chemical.

1 5. The erythrocyte of Claim 1, wherein said antigen
2 is a non-immunogenic substance found in the mammalian
3 primate circulatory system, which can become pathogenic
4 or cause adverse biological effects if present in
5 sufficient amounts.

1 6. The erythrocyte of Claim 4, wherein said antigen
2 is HIV (The AIDS virus).

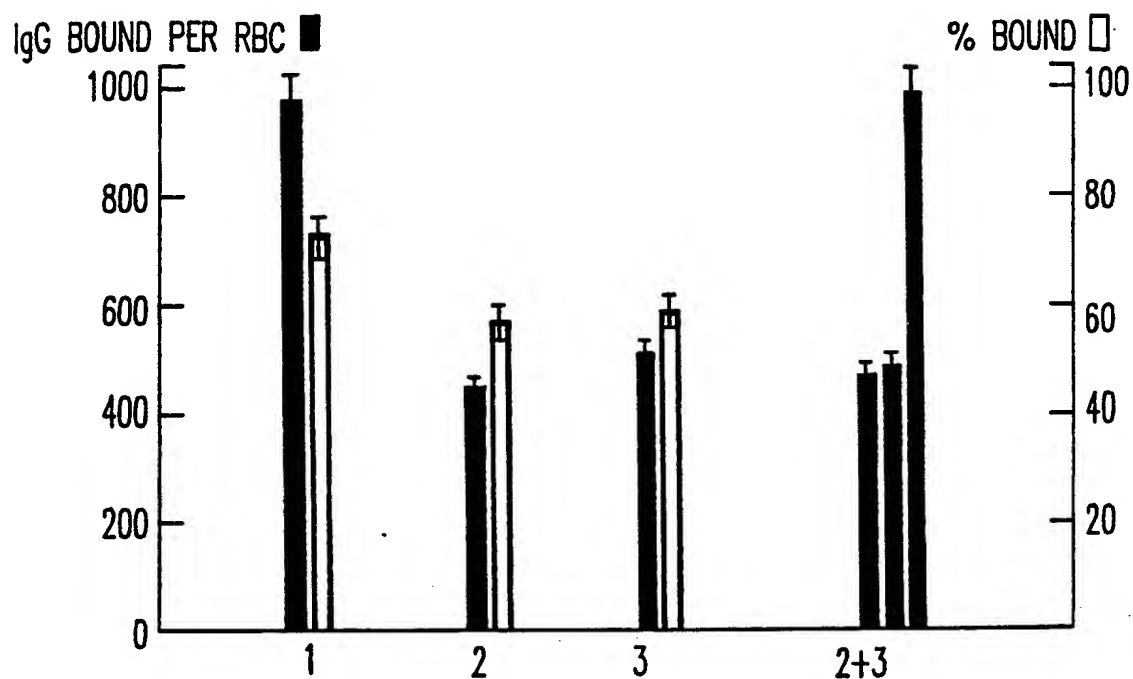
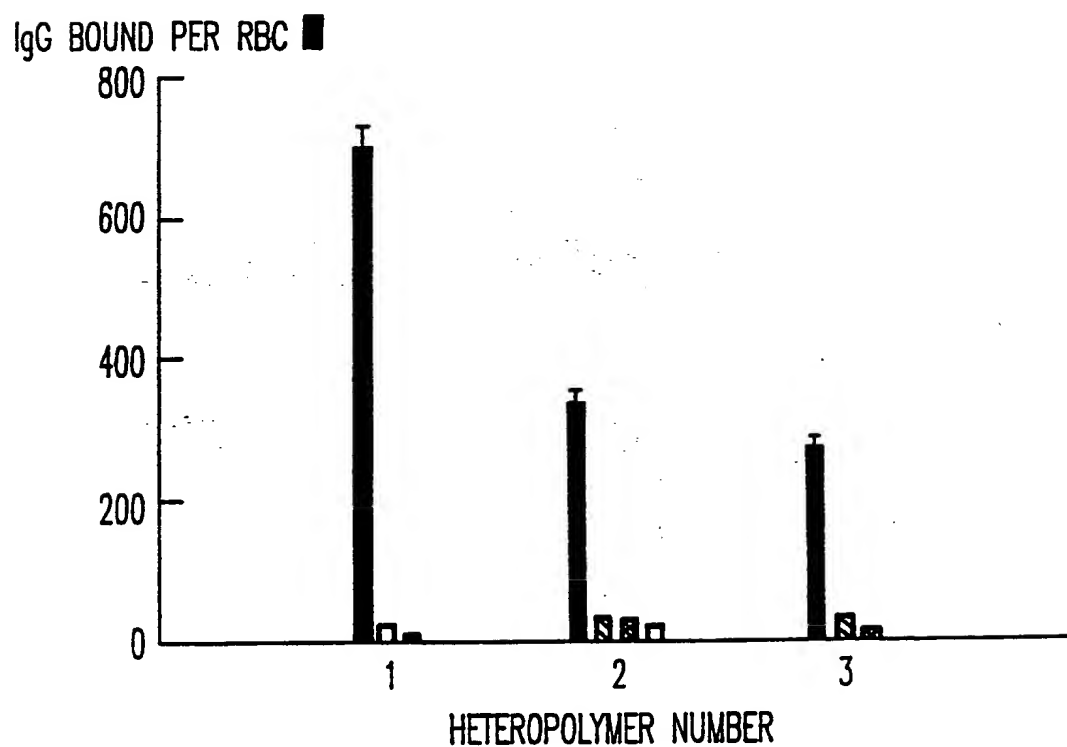
1 7. The erythrocyte of Claim 5, wherein said antigen
2 is low density lipoprotein.

1 8. The erythrocyte of Claim 1, wherein said
2 erythrocyte is a human or other primate erythrocyte.

1 9. The erythrocyte of Claim 8, wherein said
2 monoclonal antibodies are obtained from a non-human host,
3 or from human sources.

1 10. A method of therapy treating a mammalian
2 primate individual having an antigen present in its
3 circulatory system, comprising inoculating said
4 individual with a monoclonal antibody heteropolymer
5 comprising a first monoclonal antibody specific for a
6 receptor site on the surface of the erythrocyte of said
7 individual, said first monoclonal antibody being cross-
8 linked to a second monoclonal antibody specific for said
9 antigen, said inoculation being performed in sufficient
10 amounts to permit binding of sufficient antigen to
11 erythrocytes via said antibodies to reduce the amount of
12 free antigen in circulation in said individual to a level
13 below that which is cytopathic or which causes adverse
14 biological effects to said individual. The inoculation
15 may be performed either directly with said heteropolymer,
16 or indirectly by franking the patient's erythrocytes (or
17 erythrocytes from a compatible donor) with said
18 heteropolymer, followed by inoculation of the franked
19 erythrocytes.

I/II

FIG. 1a**FIG. 1b**

2 / 11

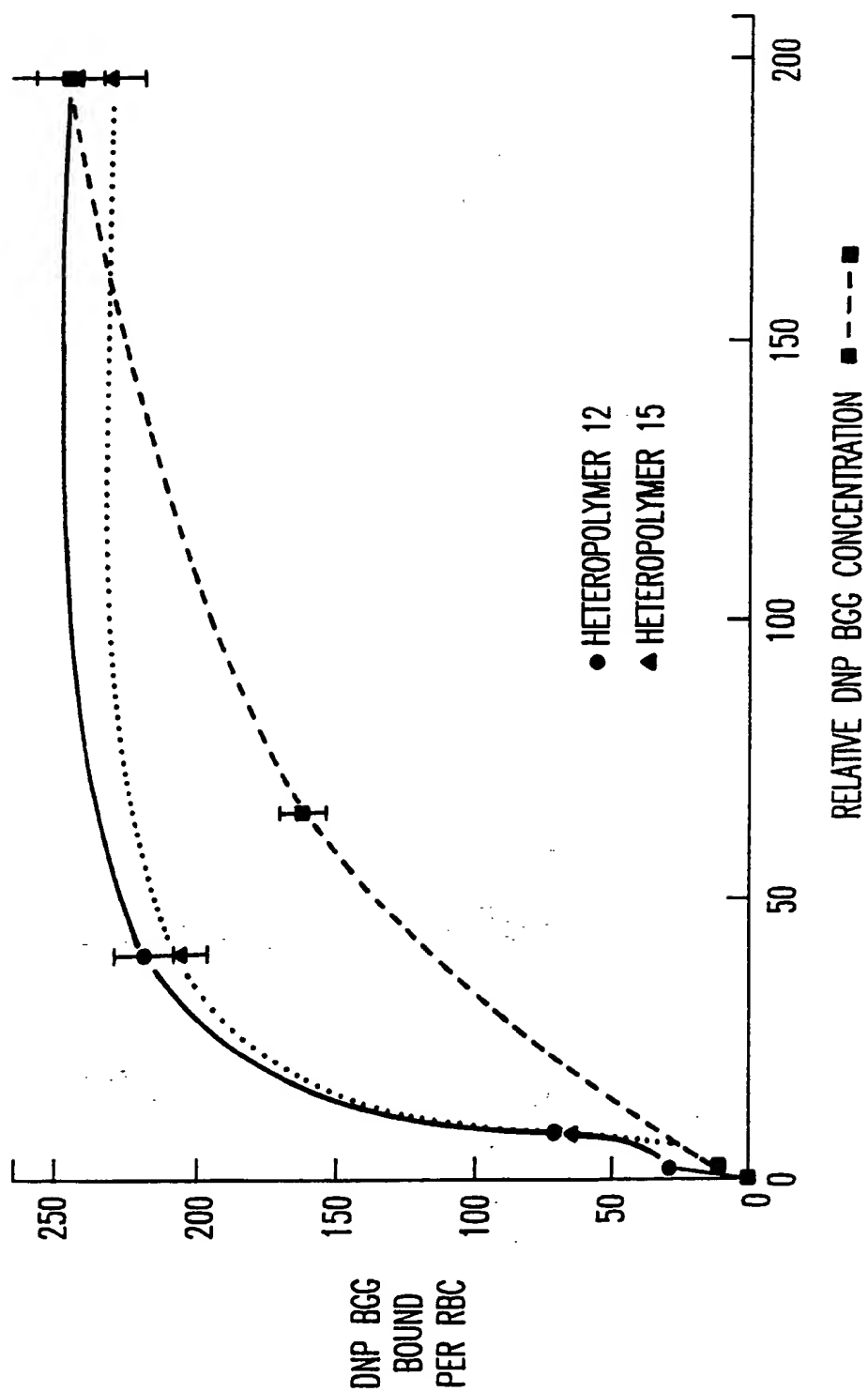


FIG. 2

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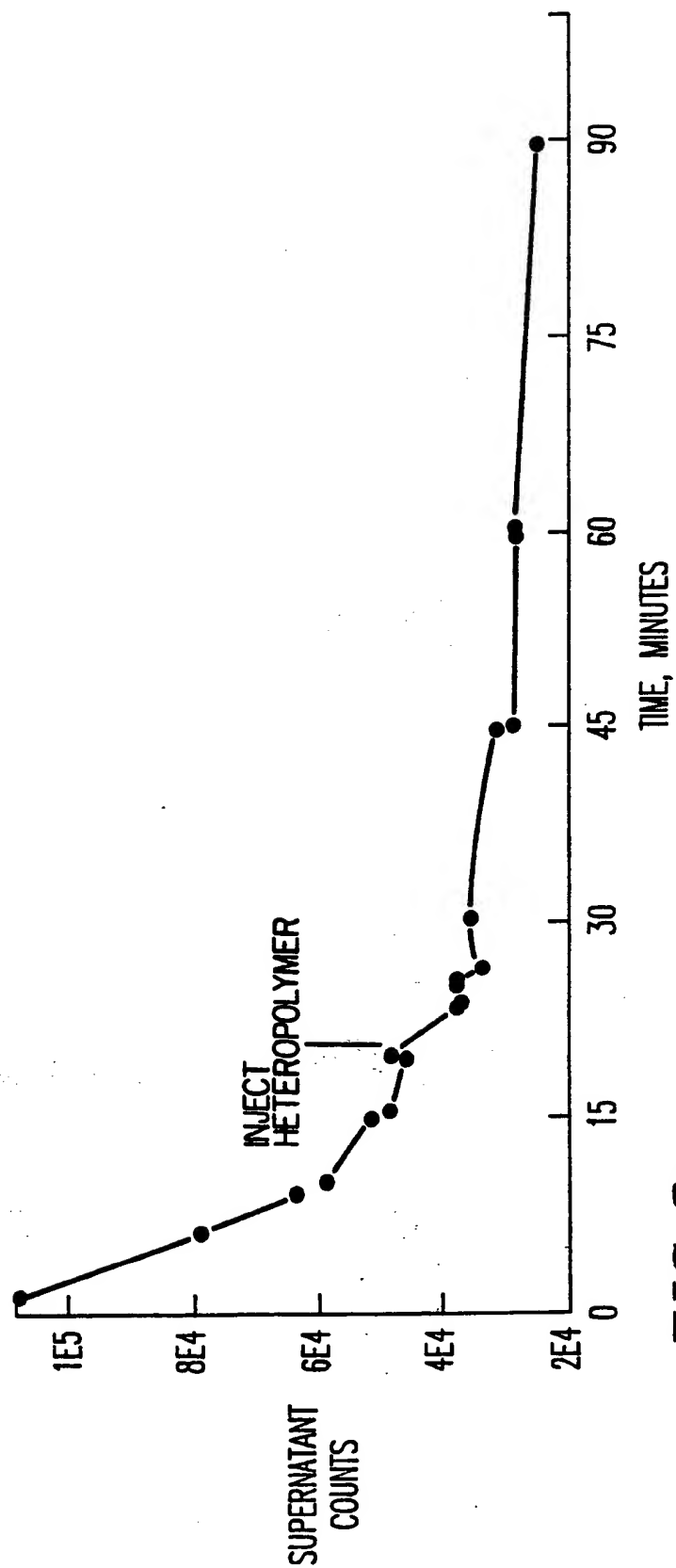
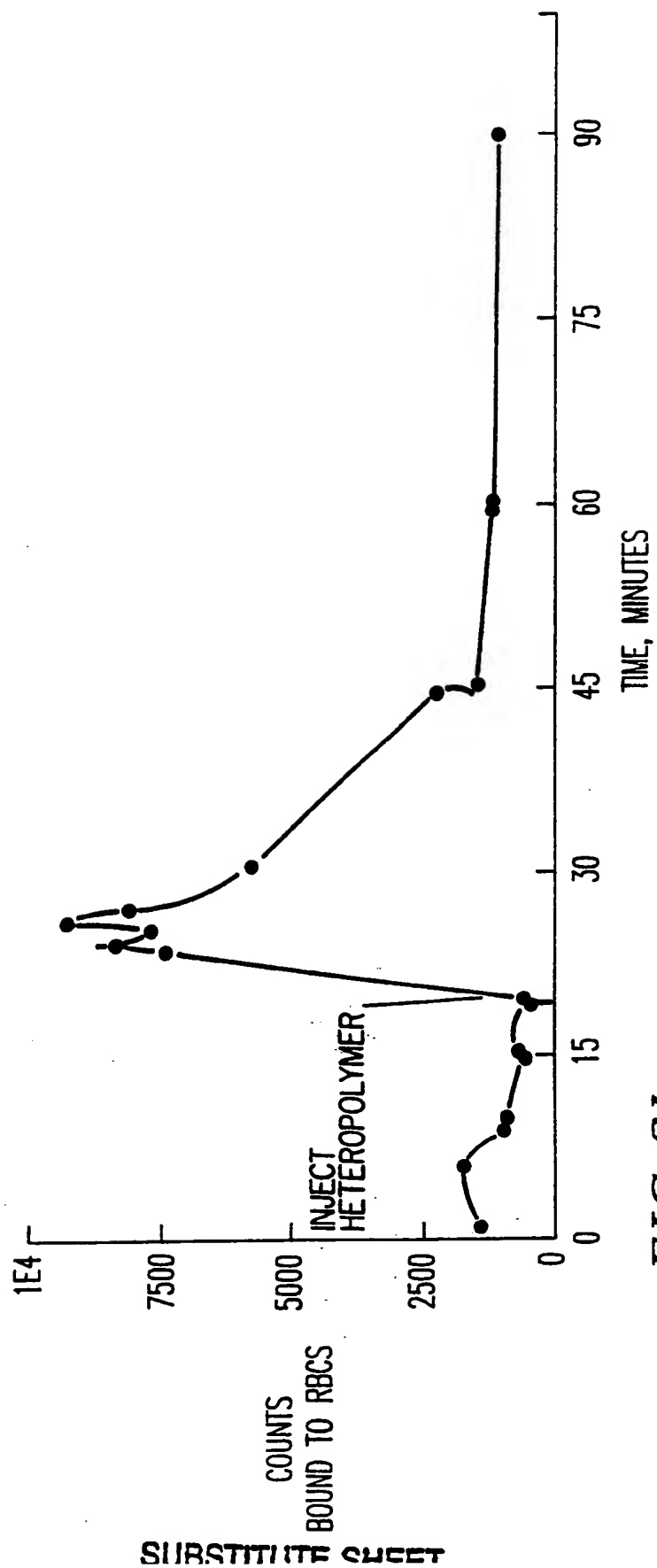


FIG. 3a

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5/11

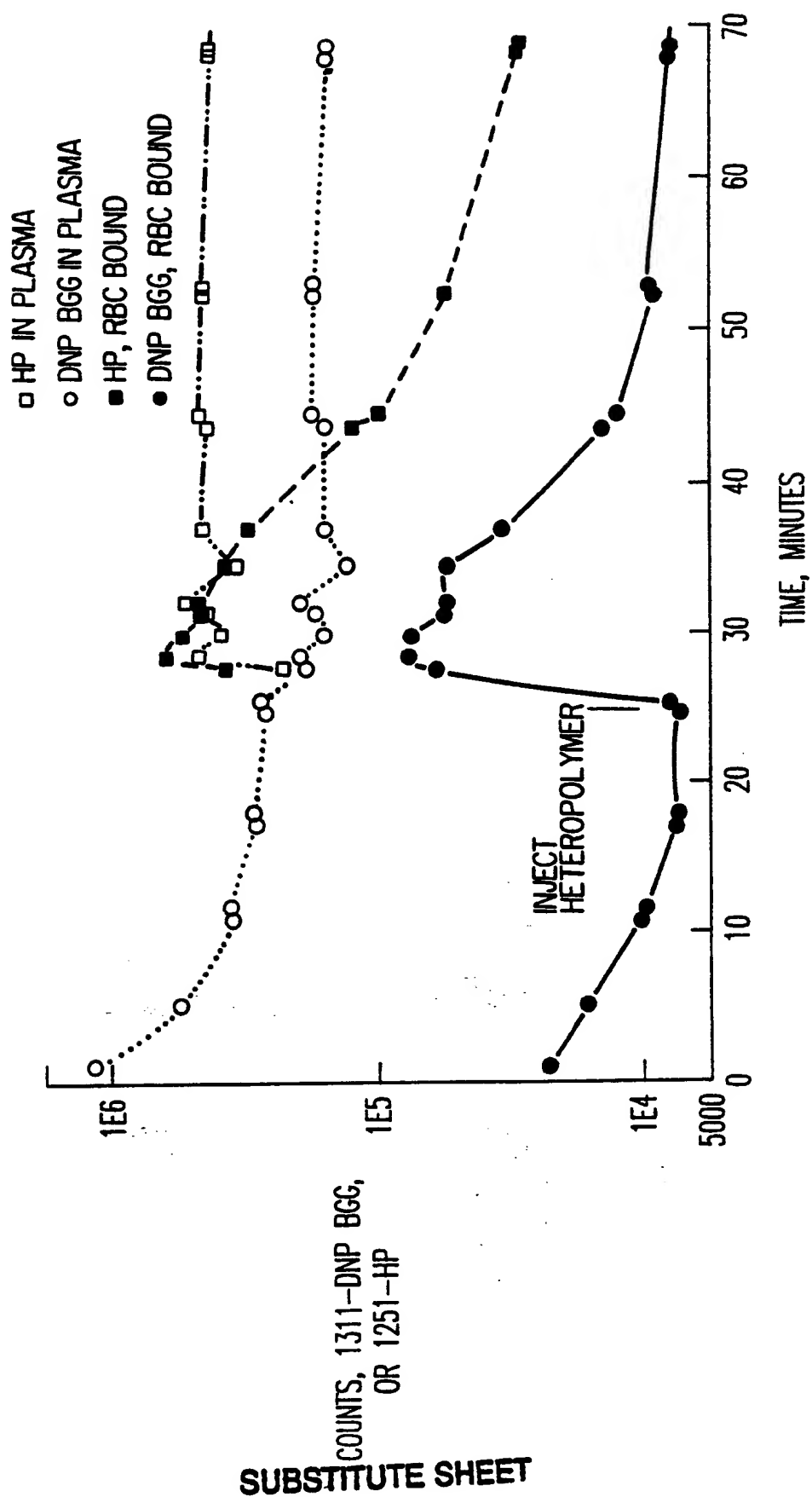


FIG. 4a

SUBSTITUTE SHEET

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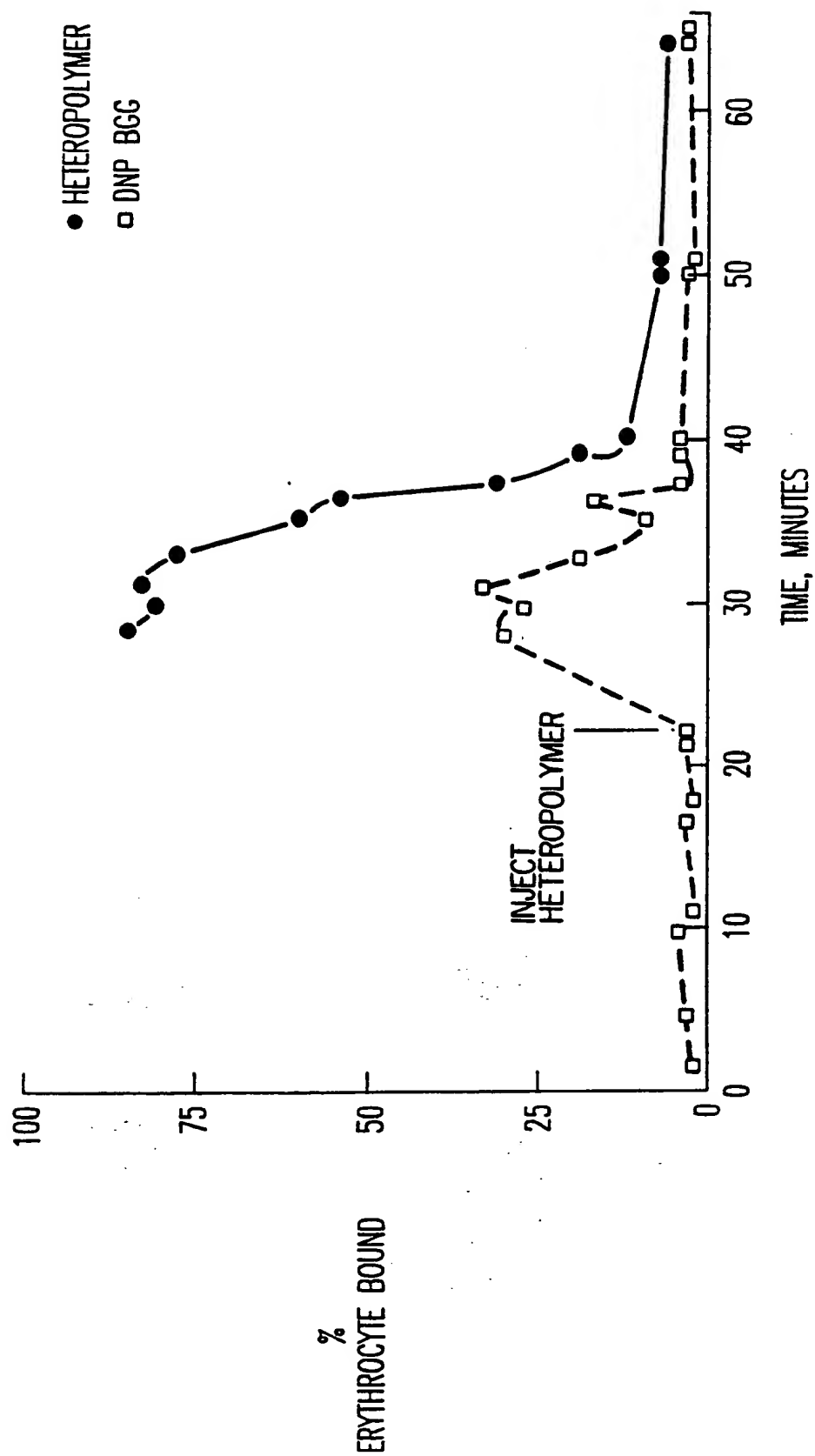


FIG. 4b

7 / II

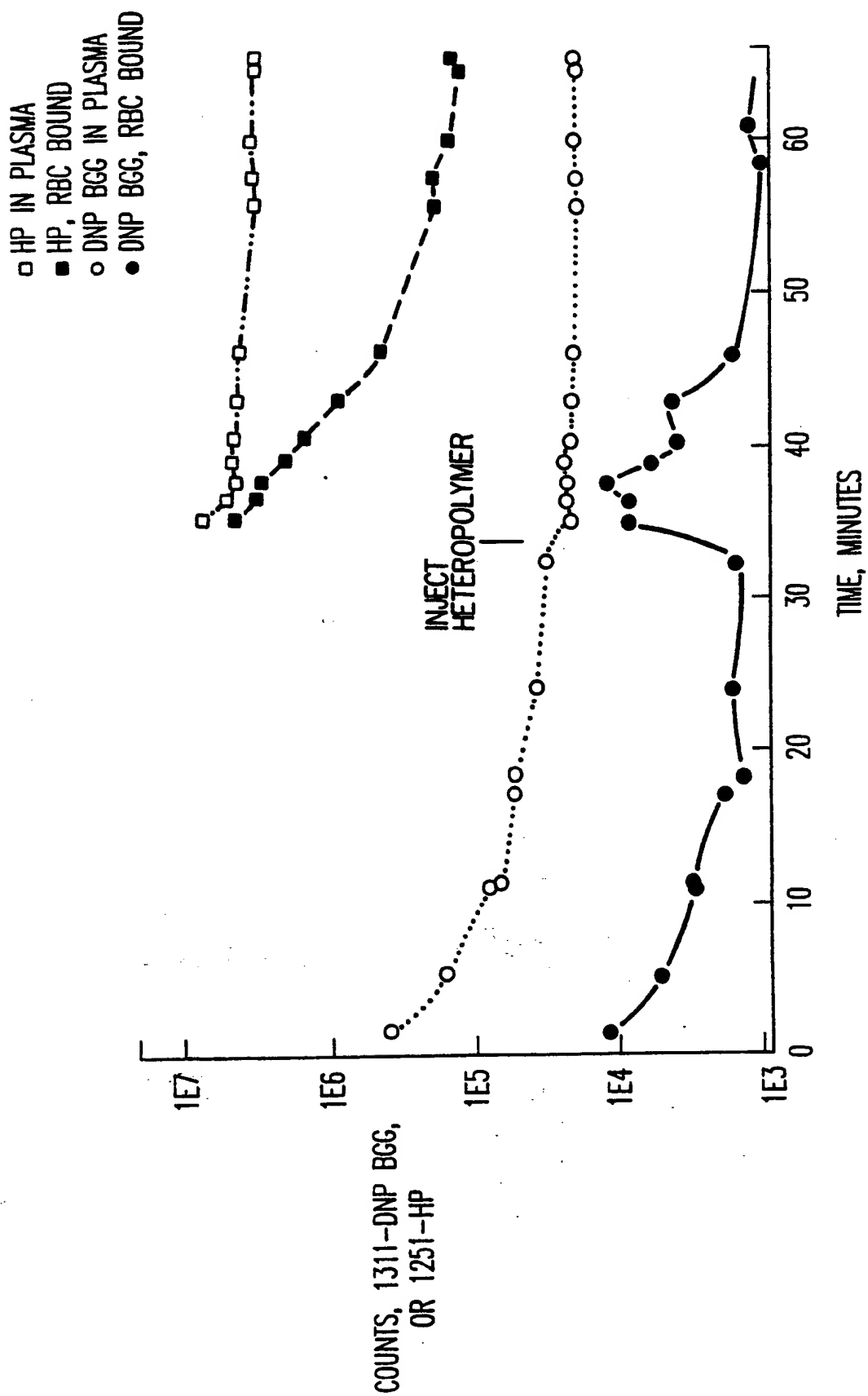


FIG. 5a

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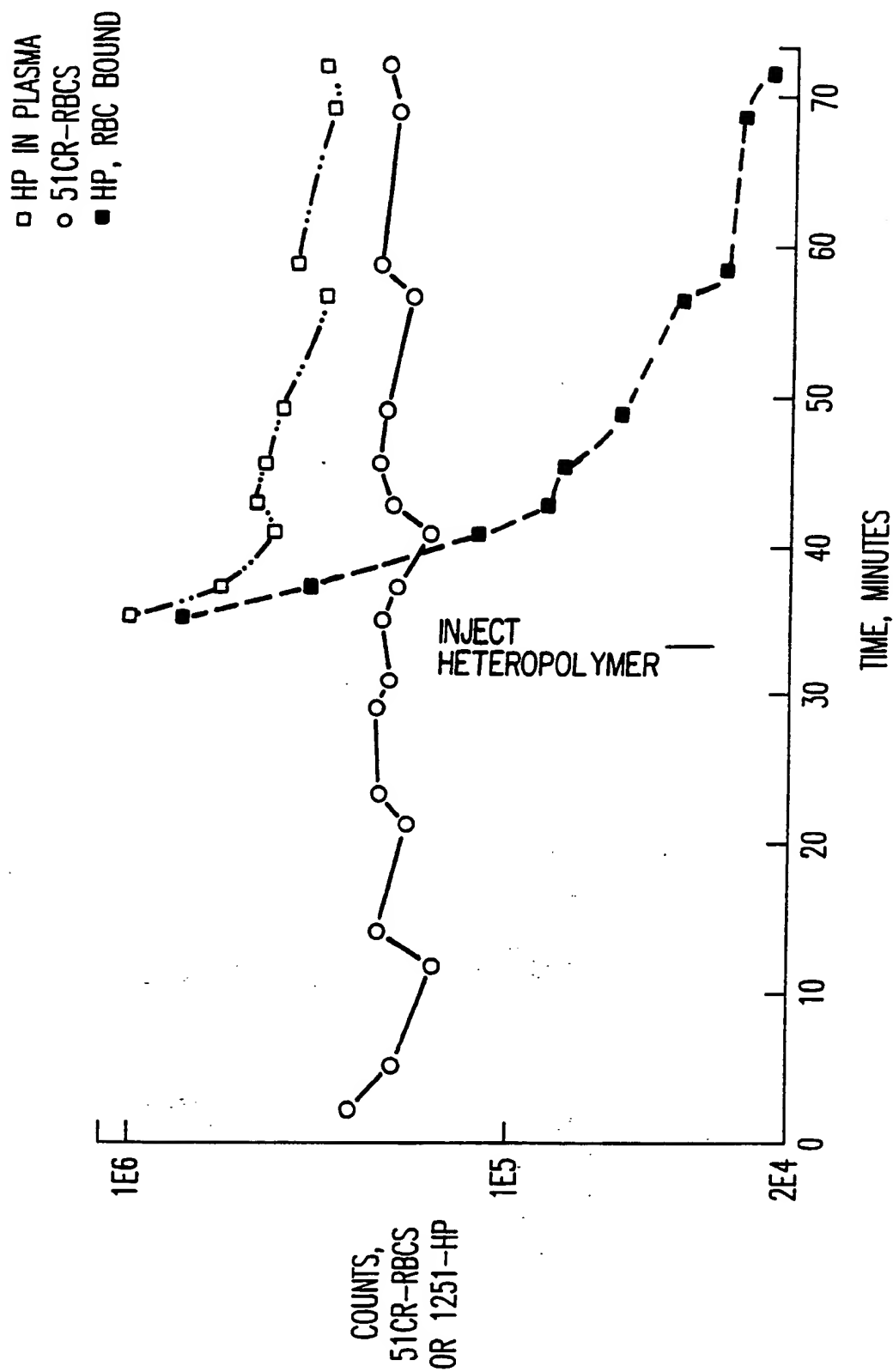


FIG. 5b

9/11

□ HP IN PLASMA
■ HP, RBC BOUND

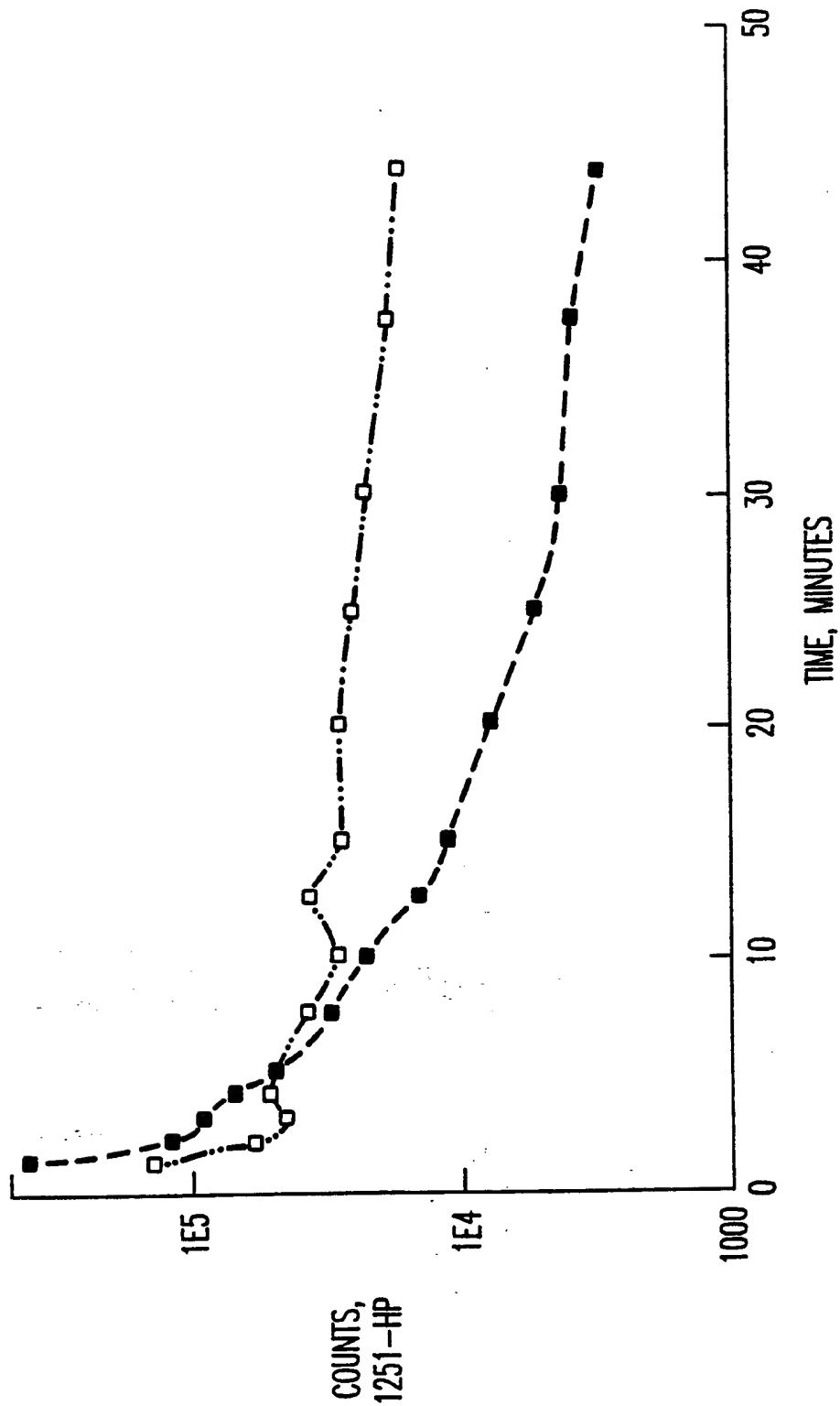


FIG. 6a

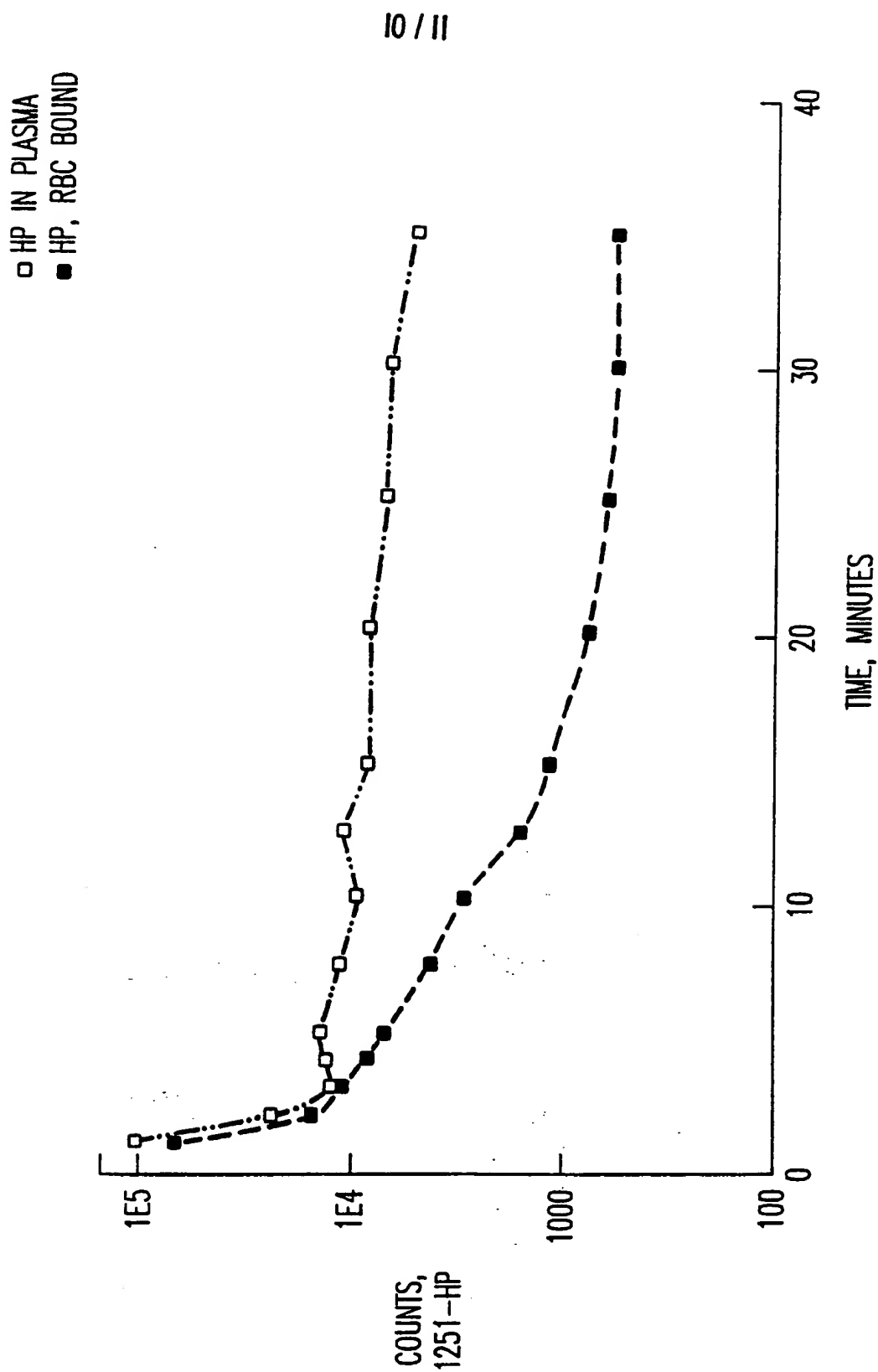


FIG. 6b

II / II

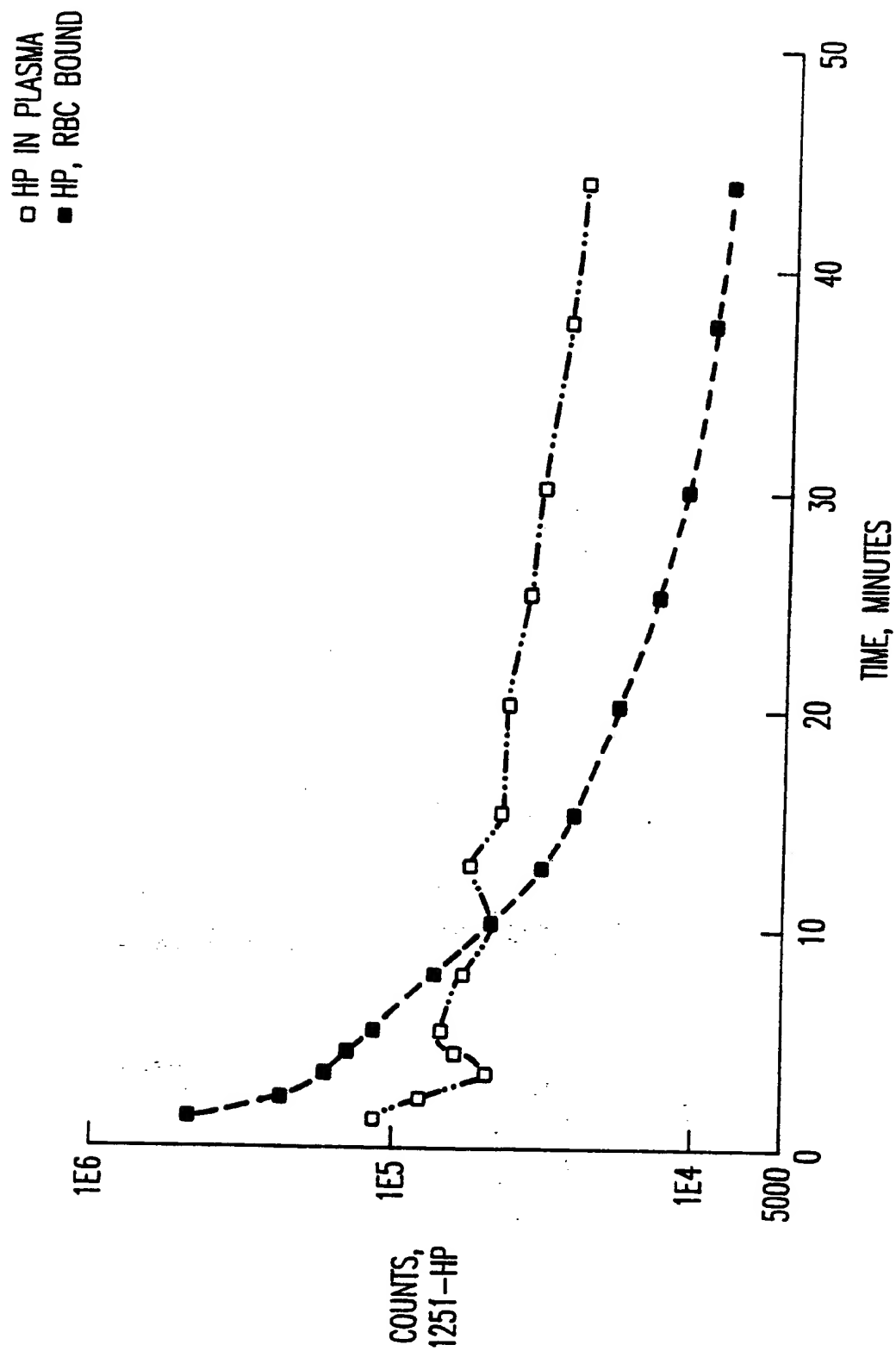


FIG. 6c

INTERNATIONAL SEARCH REP RT

International Application No. PCT/US91/07158

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 39/395 , 35/14 U.S. CL.: 435/2; 424/85.8; 604/4											
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; padding: 5px;">Classification System</th> <th style="border: 1px solid black; padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; text-align: center;">U.S.</td> <td style="border: 1px solid black; padding: 5px;">435/2; 424/85.8; 604/4</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched ⁸</div>			Classification System	Classification Symbols	U.S.	435/2; 424/85.8; 604/4					
Classification System	Classification Symbols										
U.S.	435/2; 424/85.8; 604/4										
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black; padding: 5px;">Category ¹⁰</th> <th style="border: 1px solid black; padding: 5px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 10%; border: 1px solid black; padding: 5px;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="border: 1px solid black; padding: 5px;">Journal of Experimental Medicine, Volume 160, issued December 1984. Karpovsky et al., "Production of Target-Specific Effector Cells Using Hetero-Cross-Linked Aggregates Containing Anti-Target Cell and Anti-Fc Receptor Antibodies", pages 1686, lines 6-11 and 21-22; page 1687, lines 1-10; page 1688, lines 4-22; page 1691, lines 1-10 and figure 2; page 1697, Table V and lines 14-29.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="border: 1px solid black; padding: 5px;">The Journal of Immunology, Volume 139, Number 9, issued 01 November 1987. Titus et al., "Human K/Natural Killer Cells Targeted With Hetero-Cross-Linked Antibodies Specifically Lyse Tumor Cells In Vitro and Prevent Tumor Growth In Vivo", pages 3153-3158. See Abstract.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-10</td> </tr> </table> <div style="margin-top: 10px;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁴ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </div> </div> </div>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	Journal of Experimental Medicine, Volume 160, issued December 1984. Karpovsky et al., "Production of Target-Specific Effector Cells Using Hetero-Cross-Linked Aggregates Containing Anti-Target Cell and Anti-Fc Receptor Antibodies", pages 1686, lines 6-11 and 21-22; page 1687, lines 1-10; page 1688, lines 4-22; page 1691, lines 1-10 and figure 2; page 1697, Table V and lines 14-29.	1-10	Y	The Journal of Immunology, Volume 139, Number 9, issued 01 November 1987. Titus et al., "Human K/Natural Killer Cells Targeted With Hetero-Cross-Linked Antibodies Specifically Lyse Tumor Cells In Vitro and Prevent Tumor Growth In Vivo", pages 3153-3158. See Abstract.	1-10
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Journal of Immunology, Volume 139, Number 11, issued 01 December 1987, Edberg et al. "Quantitative Analysis of The Binding of Soluble Complement-Fixing Antibody/dsDNA Immune Complexes to CR1 on Human Red Cross Cells". pages 3739-3747. See entire article.	1-10